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Reversible Unfolding of Bovine β-Lactoglobulin Mutants without a Free Thiol Group*

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Bovine β-lactoglobulin (β-lg) has been used extensively as a model for studying protein folding. One of the problems preventing clarification of the folding mechanism is the incomplete reversibility from the unfolded state, probably caused by the thiol-disulfide exchange between a free thiol at Cys-121 and two disulfide bonds. We constructed and expressed three β-lg subtype A mutants in which Cys-121 was replaced by Ala, Ser, or Val (*i.e.* C121A, C121S, and C121V). We studied the reversibilities of these mutants from urea denaturation using circular dichroism, tryptophan fluorescence, reversed-phase and gel-filtration high performance liquid chromatographies, and SDS-PAGE. The folded structure of each mutant was similar to that of wild-type β-lg. Urea-induced unfolding at pH 7.0 and 3.0 showed that although the C121S mutation notably decreases the stability, the destabilizing effects of the C121A and C121V mutations are less severe. For all of the mutants, complete refolding from the unfolded state in 8 M urea at both pH 7.0 and 3.0 was observed. Kinetics of the formation of the irreversibly unfolded species of wild-type β-lg in 8 M urea at pH 7.0 indicated that, first, an intramolecular thiol-disulfide exchange occurs to produce a mixture of species with non-native disulfide bonds followed by the intermolecular thiol-disulfide exchange producing the oligomers. These results indicate that intramolecular and intermolecular thiol-disulfide exchange reactions cause the low reversibility of wild-type β-lg especially at neutral pH and that the mutation of Cys-121 improves the reversibility, enabling us to study the folding of β-lg more exactly under various conditions.

Bovine β-lactoglobulin (β-lg),[1] one of the main protein components of cow's milk but absent in humans, is composed of nine β-strands and one short and one long α-helix (Fig. 1) (1–6). β-lg binds a variety of hydrophobic compounds including retinol and fatty acids at the hydrophobic cavity of the molecule, although the physiological function of β-lg and moreover the ligand binding are unknown (1, 6). β-lg is made of 162 amino acid residues (a molecular weight of 18,400), five of which are cysteine residues, *i.e.* Cys-66, Cys-106, Cys-119, Cys-121, and Cys-160. Cys-66 and Cys-160 form a disulfide bond near the surface of the protein molecule, Cys-106 and Cys-119 form a disulfide bond inside, and Cys121 retains a free thiol group. Although β-lg exists as a homodimer at neutral pH, it dissociates into monomers at acidic pH but still retains the native structure (7–12). Because of its stability under acidic pH conditions, β-lg is resistant to digestion in the stomach and is considered one of the allergens for human infant milk allergy (13).

In addition, β-lg shows a unique characteristic in its folding. Although β-lg is a predominantly β-sheet protein, Shiraki *et al.* (14) noticed that the addition of trifluoroethanol induces a drastic conformational change to a predominantly α-helical structure. Secondary structure predictions indicated that β-lg has a high α-helical propensity determined by local interactions (15, 16). Moreover, the refolding kinetics measured by the far-UV circular dichroism (CD) indicated the accumulation of intermediates with non-native α-helices (17). Taken together, Hamada *et al.* (17, 18) proposed that, during the refolding of β-lg, intermediates with non-native α-helices accumulate because of the strong local α-helical preference. However, nonlocal interaction and overall free energy favor the native β-structure and the slow α-helix-to-β-sheet transition produces the native β-lg.

Recent studies with H/D exchange of amide protons combined with the heteronuclear NMR analysis suggested the structure of the intermediate (19, 20). In the rapidly formed intermediate that accumulated several microseconds after the initiation of refolding, several regions showed persistent resistance to exchange. This includes the regions of β strands F, G, and H and the C-terminal helix. In addition, several residues in β strand A indicated a weak protection. The pattern of protection suggested that β strand A assumes a non-native α-helix in the intermediate (20). Thus, the intermediate is likely to contain both native and non-native structures so that the present picture of the folding of β-lg is a mixture of the hierarchical and non-hierarchical folding. However, we do not understand the role of the non-native α-helical structure in protein folding. Does it accelerate or decelerate the folding of β-lg? The exact folding mechanism of β-lg is important to understand the interplay between the local and non-local interactions, which might play a role in several biologically important processes such as the conformational transition of prion protein (21).

Despite the interesting characteristics of β-lg in protein folding, the folding experiments with β-lg have been performed mainly at acidic pH (17, 19, 20, 22, 23). One of the factors preventing the use of neutral or alkaline pH conditions is the low reversibility of the unfolded state (24–26). Irreversible denaturation has been thought to arise from thiol-disulfide

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[1] The abbreviations used are: β-lg, β-lactoglobulin; CD, circular dichroism; HPLC, high performance liquid chromatography; NaPi, sodium phosphate; Gly-HCl, glycine-HCl.

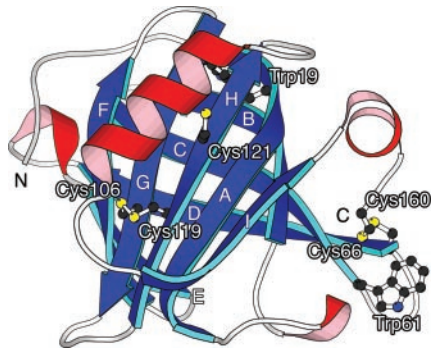

FIG. 1. **The crystal structure of β-lg.** The positions of disulfide bonds (Cys-66/Cys-160, Cys-106/Cys-119), a free cysteine residue (Cys-121), tryptophan residues (Trp-19, Trp-61), and β-strands (A–I) are indicated in the structure. The diagram was created by Molscript (42) with the Protein Data Bank code 1BEB (3).

exchange caused by the free thiol of Cys-121. Indeed, the formation of intermolecular disulfide bridges by heat denaturation at neutral pH has been reported previously (27–30). Even at pH 2.1, an incomplete recovery of CD spectra was observed in samples treated with 7 M urea (22). The proximity of Cys-121 to Cys-119 may make the thiol-disulfide exchange possible even under acidic conditions. Although site-directed mutagenesis at Cys-121 was attempted previously to prepare a species devoid of the free thiol group, a mutant without Cys-121 was not obtained with the *Escherichia coli* expression system (31).

In this study, we successfully constructed and expressed Cys-121-substituted mutants of bovine β-lg subtype A (*i.e.* C121A, C121S, and C121V). We studied their structures, resistance to urea denaturation, and reversibility from the unfolded state in 8 M urea at pH 7.0 or 3.0. We also investigated the time-dependent modification in 8 M urea causing the irreversible denaturation of wild-type β-lg. Hereby, we can state that a free thiol at Cys-121 attacks the disulfide bonds and forms incorrect pairs in an unfolded β-lg, decreasing the reversibility of wild-type β-lg, and that the mutants used here enable us to study the folding of β-lg under various conditions.

EXPERIMENTAL PROCEDURES

Plasmids for Mutagenesis—The primers used are 5′-C CTG GTC TGC CAG GCC CTG GTC AGG ACC-3′ for the C121A mutation, 5′-CTG GTC TGC CAG TCC CTG GTC AGG AC-3′ for the C121S mutation, and 5′-C CTG GTC TGC CAG GTC CTG GTC AGG ACC-3′ for the C121V mutation. These primers and their complementary primers were purchased from Sigma. We introduced site-directed mutations with these primers by the QuikChange method (Stratagene, La Jolla, CA) into previously constructed plasmids (pPIC11) containing the bovine β-lg subtype A sequence (32). To obtain a larger amount of plasmids, *E. coli* XL-1 Blue cells were transformed with the plasmids and the transformants were selected using ampicillin on LB plates. After the incubation of *E. coli* containing the plasmids in test tubes, a Wizard Plus Miniprep kit (Promega, Madison, WI) was employed to extract the plasmids. We verified the sequences of the mutants in the plasmids using a DNA sequencer, the Applied Biosystems Model 310 (Foster City, CA).

Protein Expression and Purification—Each plasmid confirmed to contain the desired sequence was linearized using the restriction enzyme AatI (Toyobo, Osaka, Japan) for *Pichia pastoris* GS115(*his4*) (Invitrogen) transformation. Electroporation of *P. pastoris* was performed with a pulse voltage of 1.7 kV using an *E. coli* pulser (Bio-Rad). The transformed cells suspended in 1 M sorbitol were spread on RDB plates without His, and colonies of His^+ transformants appeared in 2 or 3 days. Some were picked from plates of each mutant and incubated in test tubes to assess their expression. The quantity of protein expressed was checked with SDS-PAGE, and one of the strains with the highest level of expression was selected for every mutant and adopted for subsequent mass culture in a 1-liter jar fermenter. For wild-type β-lg, we used the strain constructed previously and stored as glycerol stock (12). The broth of mass culture was centrifuged, and the supernatant was filtered with 5.0-μm-pore size Millex-SV (Millipore, Bedford, MA). The filtered supernatant was diluted 5-fold with water and adjusted to pH 3.5 with concentrated HCl. Each type of β-lg in the supernatants was adsorbed by a CM-Sepharose CL-6B (Amersham Biosciences) column equilibrated with 50 mM glycine-HCl (Gly-HCl) buffer (pH 3.5) and eluted with a gradient of 0–1.0 M NaCl. The solutions with β-lg were dialyzed against dilute HCl at pH 2.5 and lyophilized. The purities of the wild-type β-lg, C121A, and C121S were very high judging from the patterns of SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. No glycosylation was detected for all of the mutants as well as the wild-type β-lg as is the case of β-lg subtype A purified from milk. On the other hand, mass spectra of C121V detected the contamination of species without 2–4 residues of the N terminus. Regardless of purification conditions, we could not remove these modified species. However, because the N-terminal deletions seemed not to affect the structure or reversibility, we used the fraction of C121V without further purification.

CD Measurements—CD spectra were acquired with a Jasco J-720 CD spectropolarimeter at 20 °C. Samples were prepared in 50 mM sodium phosphate (NaPi) buffer (pH 7.0) and contained 0.1 mg ml^{-1} of each protein for far-UV measurements using a 0.2-cm cell and 1.0 mg ml^{-1} for near-UV measurements using a 1.0-cm cell. The concentrations of proteins were determined from the absorbance at 280 nm with molar extinction coefficient at 280 nm (ϵ_{280}) calculated using: $\epsilon_{280} = 5690N_{Trp} + 1280N_{Tyr} + 120N_{S\text{-}S}$, where N_{Trp}, N_{Tyr}, and $N_{S\text{-}S}$ are the numbers of tryptophan, tyrosine, and disulfide bonds, respectively (33). The data were represented by the mean residue ellipticity, $[\theta]$.

Fluorescence Measurements—Tryptophan fluorescence spectra were measured with a Hitachi F-4500 fluorescence spectrophotometer at 20 °C. Samples contained 0.1 mg ml^{-1} of each protein and were prepared in 50 mM NaPi buffer, pH 7.0, or 50 mM Gly-HCl buffer, pH 3.0, with each concentration of urea. Measurements were performed with excitation at 295 nm using a cell with a 0.5-cm light path.

We assumed a two state mechanism of unfolding and a linear dependence of the free energy change of unfolding, $\Delta G_U^{H_2O}$, upon the urea concentration, [urea] (34). To estimate the fraction of native species, the fluorescence spectra from 305 to 400 nm were globally fitted to Equation 1,

$$F = \alpha\{f_N F_{0\,M} + (1 - f_N)F_{9\,M}\} \quad \text{(Eq. 1)}$$

where F is the fluorescence intensity at every 0.2 nm from 305 to 400 nm for a sample with each concentration of urea, $F_{0\,M}$ and $F_{9\,M}$, for 0 M and 9 M urea, respectively; f_N is the fractional population of native species, and α is a variable term for fluorescence intensity. f_Ns were plotted against urea concentration, and thermodynamic parameters were calculated by fitting to Equation 2,

$$y = \frac{(a + b[\text{urea}]) - (c + d[\text{urea}])}{1 + \exp\{-(\Delta G_U^{H_2O} - m[\text{urea}])/RT\}} + (c + d[\text{urea}]) \quad \text{(Eq. 2)}$$

where y is the observed f_N, $\Delta G_U^{H_2O}$ is the free energy change of unfolding in the absence of urea, m is a parameter for cooperativity of unfolding, and $(a + b[\text{urea}])$ and $(c + d[\text{urea}])$ are terms for the base-line dependence on urea concentration. The midpoint concentration of unfolding, c_M, is calculated by Equation 3.

$$c_M = \Delta G_U^{H_2O}/m \quad \text{(Eq. 3)}$$

The fitting was operated with the software Igor (WaveMetrics, Lake Oswego, OR).

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were carried out with a Beckman Optima XL-I analytical centrifuge. Samples for each type of β-lg were prepared in 20 mM NaPi buffer, pH 7.0, containing 20 mM NaCl with three types of protein concentrations (0.33, 0.67, and 1.0 mg ml^{-1}). The dimerization constant of monomer-dimer equilibrium, K_D, was determined by fitting C_T–r plots as described before (10–12, 35).

Refolding Yield—To measure the reversibility of unfolding, samples in 8 M urea were diluted 30-fold with buffers after a 2-day incubation at 20 °C. The refolding was monitored based on CD and tryptophan fluorescence. For the measurements of time dependence, the incubation time in 8 M urea was varied.

The reversibility of unfolding was also measured by reversed-phase HPLC and gel-filtration HPLC. HPLC experiments were performed with a Gilson HPLC system. A C_4 column (Waters, Milford, MA) and TSK-Gel $G3000SW_{XL}$ column (Tosoh, Tokyo, Japan) were employed for the reversed-phase and gel-filtration HPLC measurements, respectively. For both measurements, the flow rate was 0.5 ml min^{-1} and

TABLE I
Expression yields and dimerization constants of the wild-type β-lg and its mutants

Species	Yield	K_D[a]
	mg liter^{-1}	M^{-1}
Wild-type	170	$2.46\ (\pm 1.07) \times 10^4$
C121A	190	$1.94\ (\pm 0.49) \times 10^4$
C121S	330	$3.25\ (\pm 0.16) \times 10^4$
C121V	160	$3.19\ (\pm 1.07) \times 10^4$

[a] Errors are fitting errors.

protein elution was monitored from absorbance at 220 nm. Protein samples (3.0 mg ml^{-1}) in 50 mM NaPi buffer, pH 7.0, with 8 M urea were incubated at 20 °C. For reversed-phase HPLC, samples were diluted 30-fold and 50 μl of each diluted sample was applied to the column equilibrated with 25% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid. Proteins were eluted with a gradient of acetonitrile from 25 to 55% (v/v). For gel-filtration HPLC, samples were diluted 10-fold and 50 μl of each sample was applied to the column equilibrated with 50 mM NaPi buffer, pH 7.0, containing 100 mM NaCl.

SDS-PAGE was carried out to monitor the formation of disulfide bonded oligomers. The unfolded samples incubated in the same condition as used for the reversed-phase or gel-filtration HPLC measurements (3.0 mg ml^{-1} of each protein, 50 mM NaPi at pH 7.0, 8 M urea, and 20 °C) were diluted 10-fold with 100 mM HCl to quench the thiol-disulfide exchange reaction. For the preparation of the reduced samples, the unfolded samples incubated for 48 h were reduced with 100 mM dithiothreitol at 37 °C for 3 h and then diluted with 100 mM HCl to the same protein concentration as the non-reduced samples. 16 μl of each sample then was mixed with 4 μl of 5-fold concentrated sample buffer and applied to the gel. To obtain the intensities of peaks, an image analysis was performed with the software Image-Pro Plus (Media Cybernetics, Silver Spring, MD).

RESULTS

Structure of Mutants—We expressed the three mutants (C121A, C121S, and C121V) of bovine β-lg subtype A efficiently in methylotrophic yeast *P. pastoris*. The final yields of C121A, C121S, and C121V cultured with 1 liter of medium were ~190, 330, and 160 mg, respectively (Table I). Under the same conditions, the yield of wild-type β-lg was 170 mg (Table I), indicating that the removal of the free thiol group at Cys-121 does not affect the expression yield.

As was also shown in our previous papers (12, 14), the far-UV CD spectrum of wild-type β-lg at pH 7.0 had a minimum at 218 nm with an ellipticity of −6000 (Fig. 2*a*), showing that it is a predominantly β-sheet protein (Fig. 1). The near-UV spectrum with minima at 293 and 285 nm (Fig. 2*a*) was also consistent with the previous papers (12, 14). Far- and near-UV CD spectra of three mutants (C121A, C121S, and C121V) were similar to each other and also to those of wild-type β-lg, showing that the mutations of Cys-121 did not change the global conformation (Fig. 2*a*). In particular, the CD spectra of C121A were indistinguishable from those of wild-type β-lg. The CD spectra were also similar between the wild-type and mutant proteins at pH 2.0 (data not shown).

β-lg has two tryptophan residues, one at position 19 on β-strand A and the other at position 61 on β-strand C (Fig. 1). Although Trp-19 facing into the base of the hydrophobic pocket before the bend of the βA strand is fully buried, Trp-61 at the end of the βC strand is relatively exposed to solvent (3–6, 8). At pH 7.0 in the absence of urea, the fluorescence spectrum of wild-type β-lg had a maximum at 329.4 nm, and upon unfolding in 9 M urea, the maximum shifted to 348.6 nm with an accompanying increase in fluorescence intensity (Fig. 3*a*). On the other hand, at pH 3.0 the native form had a maximum at 329.0 nm and maximal intensity decreased upon unfolding (Fig. 3*b*). It is intriguing that the fluorescence intensity of the native form at pH 3.0 is ~1.5-fold higher than that at pH 7.0, although the exact reason why is unknown (36, 37). The β-lg

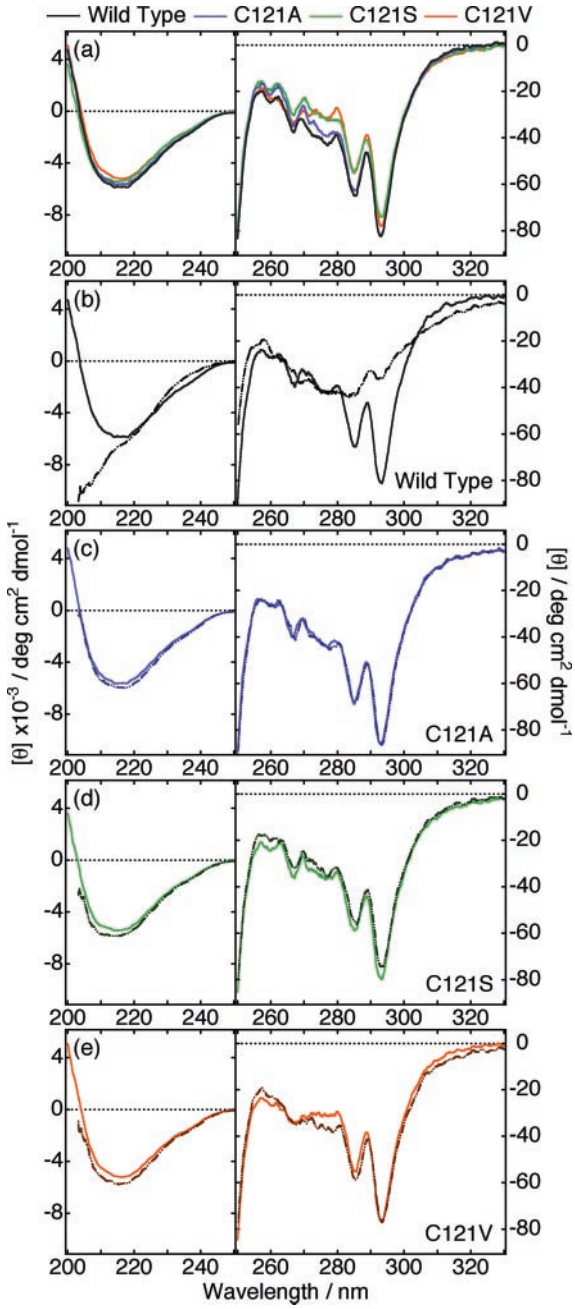

FIG. 2. **Far- and near-UV CD spectra of the wild-type β-lg and its mutants at pH 7.0 and 20 °C.** *a*, CD spectra for the wild-type β-lg and C121A, C121S, and C121V. *b–e*, comparison of the CD spectra between the native (non-unfolded, *solid line*) and refolded (*dot-dashed line*) states of the wild-type β-lg (*b*), C121A (*c*), C121S (*d*), and C121V (*e*).

mutants showed spectra very similar to those of wild-type β-lg at both pH 7.0 and pH 3.0 (data not shown). These results confirmed that the mutations do not affect the local environment of tryptophan residues. Thus, we succeeded in preparing mutants with a similar overall structure to the wild-type β-lg.

Stability of Mutants—The stability of mutant proteins was studied at pH 7.0 and 3.0 by monitoring the change in fluorescence spectra in the presence of various concentrations of urea. Each fluorescence spectrum was fitted to a sum of the native and unfolded states, and the fractions of the native species were plotted against the urea concentration (Fig. 4, *a* and *f*). Transition curves were analyzed by a two-state mechanism assuming a linear dependence of the free energy change of

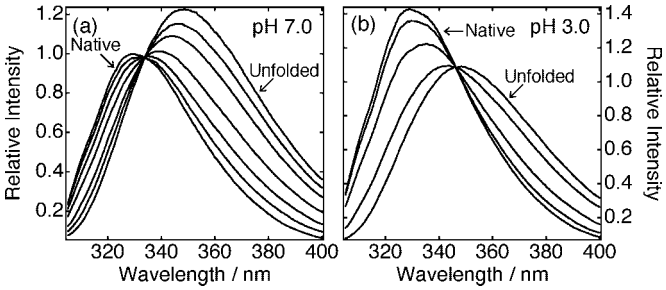

FIG. 3. **Urea-induced unfolding of the wild-type β-lg.** Unfolding was monitored with tryptophan fluorescence spectra at pH 7.0 (*a*) and pH 3.0 (*b*) at 20 °C. At pH 7.0, concentrations of urea were 0, 3.0, 3.5, 4.0, 4.5, 5.0, and 9.0 M from bottom to top at 360 nm. At pH 3.0, concentrations of urea were 0, 4.0, 4.5, 5.0, and 9.0 M from bottom to top at 360 nm. The emission intensity is relative to the intensity at 329.4 nm of the native state at pH 7.0. The spectra except in 0 M and 9 M urea were compensated for in intensity with the fitting parameter α (Equation 1).

unfolding, ΔG_U, upon the concentration of urea to obtain $\Delta G_U^{H_2O}$ (ΔG_U in the absence of urea), m (the dependence of ΔG_U on the concentration of urea), and c_M (the midpoint urea concentration) (Table II).

β-lg is more stable at acidic pH (Fig. 4*f*) than at neutral pH (Fig. 4*a*), although dimers of β-lg stable at neutral pH dissociate into monomers at acidic pH (7, 8, 10, 11). This property was conserved among the mutants. The apparent transition curves (Fig. 4, *a* and *f*) as well as the obtained thermodynamic parameters (Table II) showed that wild-type β-lg and C121A have relatively similar values at both neutral and acidic pH. On the other hand, C121S revealed a decrease of c_M and m values at both pH 7.0 and 3.0, resulting in remarkably lower values of $\Delta G_U^{H_2O}$. In the case of C121V, the c_M value was similar and slightly higher than that of the wild-type β-lg at pH 3.0 and 7.0, respectively. Because of the decrease in the cooperativity of unfolding, the $\Delta G_U^{H_2O}$ value was decreased significantly at both pH conditions.

Analytical Ultracentrifugation—β-lg is a dimer at neutral pH. We determined the dimerization constant (K_D) as a probe to monitor the subtle conformational change produced by mutation. Using the data on sedimentation equilibrium, the K_D values were calculated for the mutants (Table I). The values of the mutants were similar to the K_D of wild-type β-lg. The results showed that the monomer-dimer equilibria of the mutant β-lg species are essentially the same as that of the wild-type β-lg, confirming that the mutations did not affect the overall structure. In accordance with this finding, when retinol binding was examined by the quenching of tryptophan fluorescence upon retinol binding at pH 7.0, the mean dissociation constant was 0.054 μM for wild-type β-lg and 0.037 μM for C121S, indicating that the mutation did not affect the ligand binding.[2]

Refolding Measurements—The reversibility of denaturation in 8 M urea at pH 7.0 for 2 days was examined with the wild-type (Fig. 2*b*) and three β-lg mutants (Fig. 2, *c–e*) by monitoring far- and near-UV CD. Both far- and near-UV CD spectra indicated a much higher reversibility from the unfolded state at pH 7.0 for the mutants than the wild-type protein. For all of the mutants, there was almost no difference in CD spectra between samples incubated in the absence and presence of 8 M urea (Fig. 2, *c–e*). In marked contrast, CD spectra of wild-type β-lg after refolding indicated the presence of a considerable amount of misfolded protein (Fig. 2*b*).

Refolding transition curves after the denaturation in 8 M urea for 2 days at pH 7.0 (Fig. 4, *b–e*) or pH 3.0 (Fig. 4, *g–j*) were

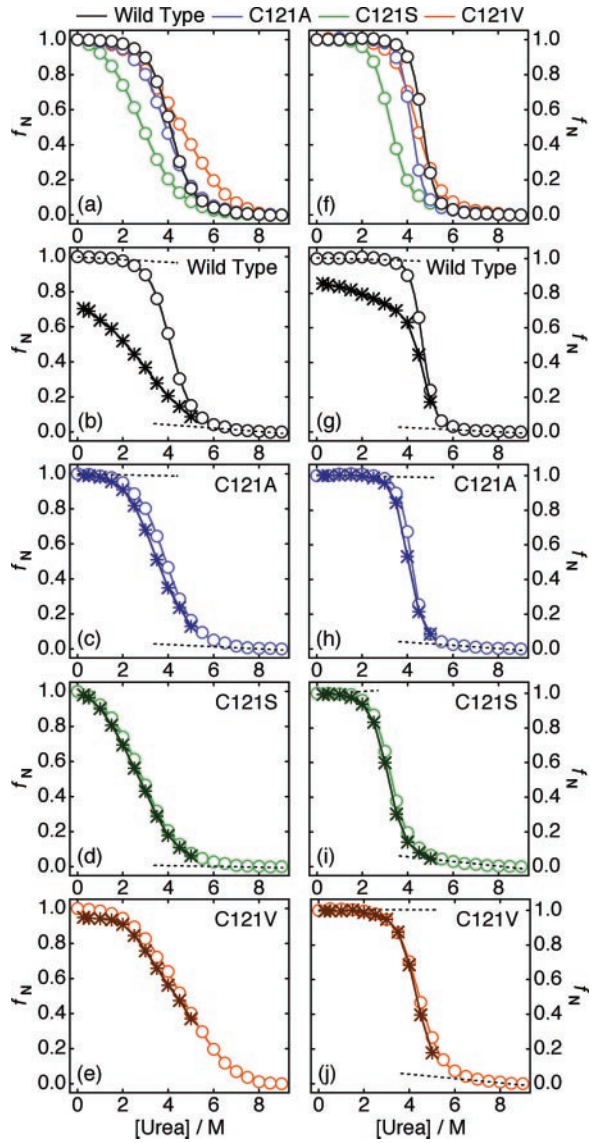

FIG. 4. **Urea-induced unfolding and refolding transitions of the wild-type β-lg and its mutants.** Transitions were monitored using tryptophan fluorescence at pH 7.0 (*a–e*) and pH 3.0 (*f–j*) at 20 °C. f_N on the basis of Equation 1 was plotted against the urea concentration. Comparison of the unfolding curves of the wild-type β-lg and its mutants at pH 7.0 (*a*) and pH 3.0 (*f*). Comparison of the unfolding curve and the refolding curve for the wild-type β-lg (*b* and *g*), C121A (*c* and *h*), C121S (*d* and *i*), and C121V (*e* and *j*). *Open circles* and *asterisks* represent the unfolding and refolding curves, respectively. Refolding was initiated after incubation in 8 M urea for 2 days at 20 °C. The lines for the unfolding transitions represent the theoretical curves on the basis of Equation 2 and the parameters shown in Table I.

constructed by plotting the fractions of native species. The wild-type β-lg showed low reversibility in particular at pH 7.0. On the other hand, at both pH 7.0 and 3.0, the refolding transition curves of the mutants agreed well with the unfolding transition curves starting with the native states, showing that the unfolding of the mutants is reversible. These results are consistent with those measured by CD, confirming that, although the reversibility is low for wild-type β-lg, it is complete for the mutants. It would be important to emphasize that, even at pH 3.0, a long incubation of wild-type β-lg in 8 M urea produces species, which cannot refold to the native state (Fig. 4*g*).

Kinetics of Irreversible Denaturation—We investigated the kinetics of the irreversible denaturation of β-lg at pH 7.0 using various methods, *i.e.* tryptophan fluorescence (Fig. 5*a*), re-

[2] C. Kalidas and C. A. Batt, unpublished results.

TABLE II
Thermodynamic parameters of urea-induced unfolding transition of the wild-type β-lg and its mutants at 20 °C[a]

pH	Species	$\Delta G_U^{H_2O}$	m	c_M
		kcal mol^{-1}	*kcal mol*$^{-1}$ *M*$^{-1}$	*M*
7.0	Wild type	21.1 (±0.6)	5.2 (±0.2)	4.09
	C121A	14.6 (±0.3)	3.8 (±0.1)	3.87
	C121S	8.2 (±0.3)	2.9 (±0.1)	2.84
	C121V	8.6 (±0.5)	1.9 (±0.1)	4.62
3.0	Wild type	41.2 (±1.2)	8.8 (±0.2)	4.67
	C121A	36.2 (±1.3)	8.7 (±0.3)	4.18
	C121S	18.3 (±0.6)	5.7 (±0.2)	3.21
	C121V	21.1 (±0.3)	4.8 (±0.1)	4.38

[a] Errors are fitting errors.

versed-phase HPLC (Fig. 5, *b* and *c*), gel-filtration HPLC (Fig. 5, *d* and *e*), and SDS-PAGE (Fig. 5*f*). We compared the wild-type β-lg and C121A, the closest mutant to the wild type. After various periods of incubation in 8 M urea at pH 7.0 and 20 °C, the protein was added to the refolding buffer (pH 7.0 and 20 °C). The extent of refolding then was examined by the methods described above. The progress of irreversible denaturation is represented by the decrease in the amount of refolded β-lg.

On the basis of a two-state mechanism, fluorescence spectra of wild-type β-lg showed a rapid decrease in reversibility and the fraction in the native state remained constant at 70% after 36 h (Fig. 5*a*). The fraction in the native state was also monitored by reversed-phase HPLC in which the non-intact forms appeared before the intact β-lg. The peak for the intact wild-type β-lg decreased more rapidly than that measured using tryptophan fluorescence, disappearing completely at 48 h (Fig. 5*b*). Because we did not assume the accumulation of intermediates with incorrect disulfide bond pairs, it is probable that such misfolded species contribute to the fluorescence spectrum after refolding, thus producing the apparent high yield of refolding monitored by tryptophan fluorescence. In contrast, the elution pattern of C121A showed a single peak independent of the incubation period in 8 M urea (Fig. 5*c*).

The gel-filtration HPLC at pH 7.0 was used to monitor the formation of oligomers. The intact β-lg was eluted at around 20.5 min as a single peak (Fig. 5*d*). We previously showed that the intact β-lg was eluted as a mixture of monomer and dimer, which are in equilibrium (10, 38). Upon incubation in 8 M urea at pH 7.0, the peak of intact β-lg decreased and a new peak appeared at 18.5 min. The peak at 18.5 min probably corresponds to the disulfide bond-linked dimer. Moreover, the formation of various species with incorrect disulfide bond pairs affects the hydrodynamic volume, resulting in a broadening of the elution pattern. The elution pattern of C121A with a single peak was independent of the incubation period in 8 M urea (Fig. 5*e*).

Oligomer formation was confirmed by SDS-PAGE in the absence and presence of reducing reagent (Fig. 5*f*). Significant amounts of dimer as well as trimer were observed in the non-reduced samples, and these oligomers disappeared in the reduced sample, showing that an intermolecular thiol-disulfide exchange reaction as well as the intramolecular reaction occurred. C121A β-lg remained monomeric even after 48 h. Similar results were obtained upon heat denaturation of the wild-type β-lg at a protein concentration of 10 mg ml^{-1} at pH 7.0 and 68.5 °C (data not shown).

The progress of irreversible denaturation monitored by various methods was compared (Fig. 6). First, the intact species monitored by reversed-phase HPLC decreased. Although tryptophan fluorescence showed a similarly rapid process, the decrease was saturated at 36 h at the high level of the native population. This is because of the contribution of the non-native states to the estimation of the native state fraction. The slow conversion of monomer to dimer and higher oligomers then was detected by gel-filtration HPLC and SDS-PAGE.

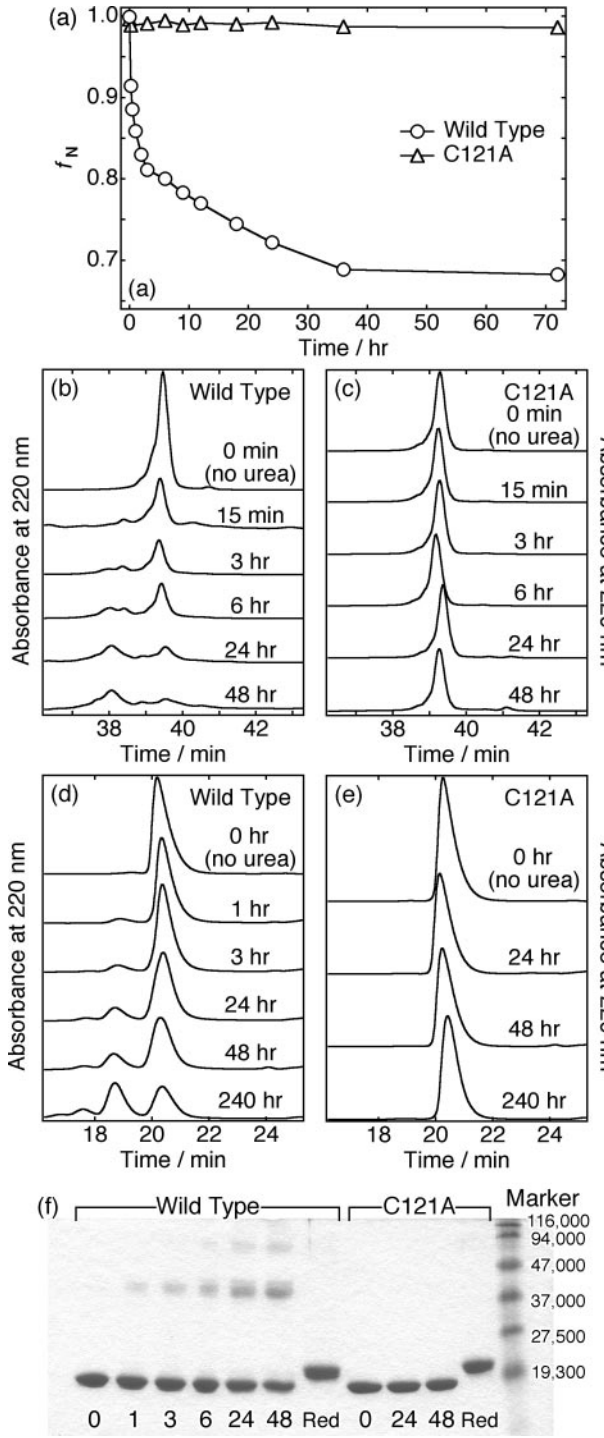

FIG. 5. **Progress of irreversible denaturation of the wild-type β-lg and C121A upon incubation in 8 M urea at pH 7.0 and 20 °C monitored by various methods.** *a*, decrease of f_N after refolding monitored using tryptophan fluorescence of the wild-type β-lg (○) and C121A (△). *b* and *c*, decrease of the native peak intensity after refolding monitored by reversed-phase HPLC for the wild-type β-lg (*b*) and C121A (*c*). *d* and *e*, formation of oligomers monitored by gel-filtration HPLC for the wild-type β-lg (*d*) and C121A (*e*). *f*, formation of oligomers monitored by SDS-PAGE for the wild-type β-lg (*left lanes*) and C121A (*right lanes*). The figures at the bottom show the incubation time in hours in 8 M urea. The samples in the lanes indicated *red* were reduced with 100 mM dithiothreitol at 37 °C for 3 h after incubation for 48 h at 20 °C in the absence of dithiothreitol.

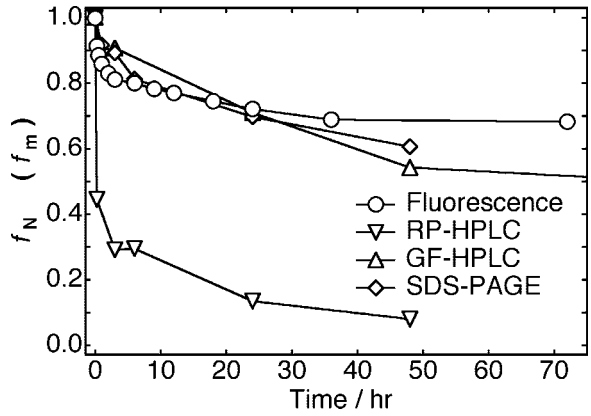

FIG. 6. **Kinetics of the decreases of the native (f_N) or the monomer (f_m) species of the wild-type β-lg upon incubation in 8 M urea at pH 7.0 and 20 °C monitored by various methods.** ○, tryptophan fluorescence taken from Fig. 5*a*; ▽, reversed-phase HPLC from Fig. 5*b*; △, gel-filtration HPLC from Fig. 5*d*; ◇, SDS-PAGE from Fig. 5*f*.

DISCUSSION

Comparison with Chemical Modification of Cys-121—We and others have studied the chemical modification of the thiol group of Cys-121 using various thiol reagents (10, 24, 25, 38–40). The reactivity of this thiol group in the native structure is very low because it is buried inside the molecule (Fig. 1). Nevertheless, various reagents have been used to specifically titrate Cys-121. The titration was often performed in the presence of a low concentration of denaturant so that the reactivity was increased. When the thiol group of Cys-121 was modified with thiol reagents, the structure of β-lg was affected, depending on the size and properties of the group introduced. Modification of β-lg by 2-mercaptoethanol or mercaptopropionic acid, which both are smaller molecules, resulted in the destruction of the dimer without a loss of the rigid native structure, although the stability of the native structure was decreased significantly (24). In the case of 5,5′-dithiobis(2-nitrobenzoic acid) (10) or tetramethylrhodamine (38), the dissociation into a monomer was coupled with significant disordering of the protein structure both at pH 7 and pH 3. The conformation of β-lg modified with 4-(*p*-dimethylamino-benzeneazo)-phenylmercuric acetate was not examined (40). Considering its size, the modified β-lg would assume a largely denatured state. In these studies in which Cys-121 of β-lg was chemically modified, improved reversibility from denaturation by heat or denaturants was noted.

Our mutants were superior to chemically modified β-lg. First, the structures of these mutants were indistinguishable from that of the wild-type as assessed with far- and near-UV CD spectra and tryptophan fluorescence spectra. Second, the dimerization constant, which is considered to be sensitive to subtle conformational changes, was independent of the mutations. Moreover, retinol binding to C121S was similar to that to wild-type β-lg.[2] These results indicate that the mutational effects introduced here were small enough not to significantly perturb the native structure of β-lg.

On the other hand, we observed notable destabilizing effects on the native structure, which varied depending on the mutations with the order as follows: C121S > C121V > C121A. The destabilizing effects can be explained by the properties of substituted amino acids. Cys-121 is located in the tightly packed hydrophobic environment (Fig. 1). Consistent with this finding, the reactivity of the thiol group is found to be very low when monitored using thiol reagents such as 5,5′-dithiobis(2-nitrobenzoic acid). The serine side chain is less hydrophobic than cysteine side chain, although the two are similar in size. The introduction of serine results in destabilization of the molecule because of the introduction of a polar group into the hydrophobic environment. On the other hand, the valine side chain is more hydrophobic and larger than the cysteine side chain. The decreased stability of C121V is predominantly because of the steric hindrance of the bulky side chains. Nevertheless, we argue that the dimerization constant of C121V (3.2×10^4 M^{-1}) is similar to that of wild-type β-lg (2.5×10^4 M^{-1}), indicating the native-fold of the C121V mutant (Table I).

Biological Implication—It would be useful to address the biological implication of the results. No definite biological function has been ascribed to β-lg, although several proposals have been suggested such as a retinol transporter from mother to neonate, a facilitator of fatty acid uptake, or a modulator of β-galactosyl transferase (1, 2). Recently, the function associated with the mother rather than the child has been focused from the sequence similarity of β-lg to glycodelin, a lipocalin expressed in large quantities in the first trimester of human pregnancy in the endometrium (1, 6). β-lg has been suggested to be a protein, which was crucial to the function of the endometrium during the early stages of pregnancy. In the context of the biological function, it is intriguing that β-lg is more stable at pH 3 than at pH 7 (Fig. 4 and Table II). The increased stability of β-lg at pH 3 is consistent with the role as a transporter of retinol to neonate through intestine, because β-lg is stable in stomach and then digested in intestine releasing the bound retinol. Intramolecular thiol-disulfide exchange reaction occurs upon unfolding, whereas it does not the native state. Thus, the partial denaturation of β-lg caused by digestion may promote the thiol-disulfide exchange reaction, producing the irreversibly denatured state and consequently accelerating the digestion. Irreversible denaturation coupled with thiol-disulfide exchange is particularly important for the heat treatment of milk in the dairy industry.

CONCLUSION

Bovine β-lg has been used extensively as a model to study the conformation and folding of proteins. However, because of the thiol-disulfide exchange reaction and consequent decrease in reversibility, most detailed refolding studies have been performed under acidic conditions. Still, the thiol-disulfide exchange reaction is likely to occur upon unfolding since Cys-121 is located very close to Cys-119, which forms a disulfide bond with Cys-106 (Fig. 1). Although β-lg species without such a free thiol group are known in several non-ruminants including the horse (41), the large amount of information accumulated with bovine β-lg favors the continuation of studies with bovine β-lg. The C121A mutant, showing similar characteristics to the wild type but with complete reversibility from the unfolded state, will be particularly useful to clarify the folding mechanisms of β-lg in which the α-helix-to-β-sheet transition, as observed for several important biological processes, might play a significant role. We expect that the folding kinetics of various mutants with increased or decreased α-helical propensity gives further insight into the mechanism of the α-helix-to-β-sheet transition.

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Reversible Unfolding of Bovine β-Lactoglobulin Mutants without a Free Thiol Group

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